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PRINCIPAL INVESTIGATOR: Marijke Mol, Ph.D.

CONTRACTING ORGANIZATION: TNO Prins Maurits Laboratory
2280 AA Rijswijk, The Netherlands

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Abstract

This study is aimed to bring about adequate leads for therapy against sulfur mustard (HD) by broadening our perception of the compound's mechanism of action. Results of experiments with the human skin *ex vivo* model show that apoptosis and metalloprotease activity are key elements in HD-induced skin pathogenesis and that intervention in these two processes was successful in resisting microvesication and impairment of epidermal cells. The observation that pancaspase inhibitor and metalloprotease inhibitor can fully prevent microvesication in the human skin *ex vivo* model if applied 6 h and 18 h, respectively, after exposure to HD opens perspectives for non-urgent cure of HD casualties. Our results indicate that microvesication in HD-exposed human skin *ex vivo* is not caused by MMP-2 activity or by reduced expression of the adhesion molecule laminin-5. The application of a proteomic approach in the search for mechanisms of action of HD has proven to be a valuable tool: involvement of apoptosis and of phosphorylated HSP27 in the cellular response towards HD was discovered. Furthermore, keratins appear to be predominant targets for alkylation by HD. Only a few other proteins are alkylated. In conclusion, the current studies in the human skin *ex vivo* model yielded general mechanistic themes that will also apply to injury induced by HD in other organs and contribute in that way to progress in the search for medical countermeasures against HD-damage in multiple organs.

1 Contents

2 Outline of the report

This report describes the results of the research project DAMD17-02-1-0206. It is a compilation of the experimental work that has been performed in the past three years. Part of the work has been described in annual reports (Mol, 2003, 2004) and symposium proceedings (Mol et al., 2003, 2004, 2005). Therefore, the present report includes the conclusive outcome of the entire study as well as some new results that were obtained in the third year.

The project was aimed to bring about adequate leads for a causal therapy against blister formation in human skin upon exposure to sulfur mustard (HD). Although the threat of a hostile attack with this vesicating agent currently seems to be low, use of vesicants by terrorists against unprotected men on peace-keeping missions or against innocent civilians can not be excluded as long as large stockpiles of HD are present in the world. Upon unprotected exposure to HD, damage to the skin, eyes and lungs will probably occur. Iranian victims of the Iraq hostilities in 1987/1988 are archetypical for the long term consequences on health if no adequate treatment can be given upon exposure. Understanding of the pathogenesis is believed to be essential for the selection of effective therapeutics. It is plausible that analogous mechanisms lie behind the pathological symptoms in skin, eyes and lungs caused by HD. Therefore, the results of the present study in which the *ex vivo* human skin model has been used to explore causing mechanisms and potential lead compounds for therapy, ultimately contribute to progress in the search for medical countermeasures against HD-damage in multiple organs.

The study is divided into three items, which correspond to tasks that are described in the research proposal. Firstly, the effectiveness of several agents to prevent epidermal-dermal separation was examined in human skin pieces that were exposed to saturated HD vapor. Their therapeutic value was evaluated by qualitative histological observations. The compounds that were investigated are six synthetic inhibitors of matrix metalloproteases (MMPs): BB94, TAPI-2, MMP inhibitor II, MMP inhibitor III, MMP2/MMP9 inhibitor and Ilomastat; a furin inhibitor; an urokinase-type plasminogen activator (uPA) inhibitor; a tyrosine kinase inhibitor; three caspase inhibitors: pancaspase inhibitor, caspase-8 inhibitor and caspase-9 inhibitor. For each of these compounds the lowest effective concentration for prevention of epidermal-dermal separation following HD exposure was determined. For two representative compounds it was examined how long time might pass between HD exposure and the start of application without loss of the protective effect. Secondly, the involvement of MMP-2 in epidermal-dermal separation was investigated by immunostaining in human skin pieces that were exposed to saturated HD vapor. Since immunostaining does not discriminate between the inactive and the active form of the enzyme, in situ zymography was applied to assess MMP-2 activity. Additionally, the fate of laminin-5 (Ln-5) during the development of microvesication has been examined. Ln-5 is an anchoring protein of the skin basement membrane (SBM) and a target for degradation by MMPs. Finally, the mechanism of HD-induced pathogenesis was investigated by studying the differential expression of proteins extracted from control and HD-treated cultured human epidermal keratinocytes (HEK) using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Comparison of the protein patterns yielded several protein spots that showed an obvious change in expression. 2D-PAGE was also used to analyse the formation of HD-protein adducts in HEK after exposure to ^{14}C-labeled HD.

Not all tasks that were described in the research proposal have been performed in the current study. Taking into account that tasks that were mostly expected to deliver new clues on therapy should deserve priority, we have focused on Tasks 1, 2, and 6. In view of the

results obtained from Task 1 and Task 2, indicating that production and degradation of hemidesmosomal proteins are of low importance in the process of microvesication, performance of Task 3 and Task 4 has become no longer relevant. On the other hand, as our results gradually were pointing out the significance of kinase signaling pathways, investigation of the phosphorylation status of hemidesmosomal proteins as described in Task 5 happened to be quite opportune. Regrettably, forced by lack of time, this task has not been carried out.

3 Body of work

3.1 *Introduction*

3.1.1 *Effectiveness of potential therapeutic agents to prevent microvesication*

Loss of the attachment competence of the SBM is postulated to facilitate HD-induced vesication of the skin and disruption of structures that effectuate the tight contact between the epidermal and dermal layer is thought to be causative. As a working hypothesis, it was assumed that destabilization of the SBM is caused by a disturbed balance between production and degradation of proteins that are involved in epidermal-dermal attachment. Protein production could probably become reduced as a result of epidermal cell death upon exposure to HD, after which protein degradation by MMPs is no longer counterbalanced by newly secreted proteins. This postulation motivates aimed intervention with drugs that prevent the onset of cell death and/or inhibit MMP activity.

It is known that proteolysis of SBM proteins is a major factor leading to changes in the SBM (Amano et al., 2001). SBM proteins are proteolytically cleaved by members of the metzincin subgroup of the superfamily of zinc proteases. This subgroup includes MMPs and ADAM(a disintegrin and metalloprotease)s, which share considerable structural homology in their active site region and are involved in normal turnover of extracellular matrix (Seals and Courtneidge, 2003). Ln-5 and collagen XVII are main adhesion proteins of epithelial cell hemidesmosomes, which mediate attachment of keratinocytes to the underlying SBM (Aumailley et al., 2003). Processing of the Ln-5 γ2-chain by MMP-2 and MT1-MMP induces epithelial cell migration (Gianelli et al., 1997; Koshikawa et al., 2000), implying loosening of keratinocyte anchorage to the basement membrane. Collagen XVII is cleaved by TNFα converting enzyme (TACE) (Franzke et al., 2002). TACE is a well-studied member of the ADAM family which primarily releases soluble signaling molecules and receptors from cells. For example, TACE processes the membrane-bound precursor of TNFα into the active soluble cytokine.

In the present study, various broad spectrum hydroxamate-based inhibitors that inhibit MMPs as well as TACE were tested on their potential to reduce HD-induced microvesication by preservation of SBM proteins. It was also investigated whether MMP activity could be brought down by the inhibition of two enzymes that act upstream in the proteinase-cascade, i.e., furin and uPA. Furin is a member of the proprotein convertase family of Ca^{2+}-dependent serine proteases and processes pro-MT1-MMP as well as TACE into their active forms (Yana and Weiss, 2000; Peiretti et al., 2003). uPA is a serine-proteinase that might be important in the activation of proMMPs via the plasminogen activator/plasmin system (Keski-Oja et al., 1992; Nagase and Woessner, 1999). Furthermore, herbimycin A, a tyrosine kinase inhibitor, has been evaluated for its therapeutic value. The rationale was the suggestion that protein tyrosine phosphorylation is involved in the activation of MMP-2 as it modulates MT1-MMP activity (Li et al., 1998). Others supposed that enhanced tyrosine phosphorylation of integrin β4 results in disruption of hemidesmosomes (Mainiero et al., 1997; Mariotti et al., 2001).

When cells are unable to repair DNA damage following treatment with genotoxic agents, they respond by initiation of apoptosis. The extrinsic as well as the intrinsic pathways leading to apoptosis, may be activated, occurring via membrane death receptors or via endogenous mitochondrial damage, respectively. Caspases are central acting proteins in the apoptotic process. Triggering of the death receptor pathway results in activation of caspase-8 (Curtin and Cotter, 2003). The mitochondrial pathway involves massive release of cytochrome c from mitochondria, which leads to activation of caspase-9 (Bras et al., 2005). Once activated, the initiator caspases, i.e., caspase-8 and caspase-9, can activate a group of effector caspases, such as caspase-3, which are responsible for the proteolytic cleavage of many intracellular proteins, resulting in the morphological and biochemical changes associated with apoptosis. Own studies (this report) and reports by others have learned that apoptosis is involved in HD-induced epidermal cell death (Rosenthal et al., 2003). Thus, it was tested whether a blockade of the apoptotic process by inhibition of all caspases has beneficial effects on the maintenance of epidermal-dermal attachment. To assess the contribution of each of the two pathways to the HD-induced apoptotic process, specific inhibitors for caspase-8 and caspase-9 were used. Furthermore, the ultimate time point was determined on which therapy with pancaspase inhibitor could be started.

3.1.2 *The involvement of MMP-2 and Ln-5 in HD-induced microvesication*

MMP-2 is thought to play an important role in epidermal-dermal separation induced by HD, since this enzyme is considered to be involved in cleavage of several proteins in the SBM (Kähäri and Saarialho-Kere, 1997). Processing of the Ln-5 γ2 chain by MMP-2 has been described (Gianelli et al., 1997; Koshikawa et al., 2000). It is assumed that following HD-exposure enhanced expression and/or activity of MMP-2 along the SBM will contribute to weakening of the epidermal-dermal junction.

Ln-5 is a major component of the SBM and has biological relevance in epidermal-dermal adhesion, as genetic mutations affecting Ln-5 have been identified in patients with junctional epidermolysis bullosa (Nakano et al., 2002). Ln-5 mediates adhesion of the epidermal cells to the underlying dermal tissue via integrin α6β4 in hemidesmosomes and a reduced expression of this integrin ligand will thus destabilize epidermal-dermal attachment. It is hypothesized that following HD-exposure the amount of Ln-5 in the SBM becomes reduced due to impaired secretion of new proteins and cell death. To understand the involvement of MMP-2 and Ln-5 during the development of microvesication in human skin pieces that were exposed to HD vapor, the expression of these proteins was assessed by immunohistochemical staining at various time points post-exposure. Furthermore, the expression of Ln-5 was investigated in skin pieces that were treated with TAPI-2 or with pancaspase inhibitor to see whether these compounds, that fully prevent microvesication, have effect on Ln-5 expression. Since the MMP-2 antibody that is used in these experiments does not discriminate between the inactive and the active form of the enzyme, *in situ* zymography was applied to assess gelatinase activity.

3.1.3 *Analysis of the mechanism of action of HD by a proteomic approach*

Proteomic analysis is a valuable tool to gain better knowledge of the critical cytotoxic events involved in the process of pathogenesis evoked by HD. To monitor the cellular response to HD exposure in time, a comparative analysis has been made of protein profiles expressed in control and HD-exposed HEK at various time points up to 42 h. In the protein profile of HD-exposed HEK, many fragments of keratins 14, 16 and 17 were observed. Since fragmentation of cytoskeletal proteins is related to apoptosis (Oshima, 2002), it was tested

whether formation of these fragments could be prevented by inhibition of certain caspases Furthermore, 2D-PAGE was applied to analyse the formation of ^{14}C HD-protein adducts in cultured HEK.

3.2 Experimental methods

3.2.1 *HD synthesis*

HD was synthesized at TNO-Prins Maurits Laboratory and has a purity of at least 97%. ^{14}C-HD was prepared according to Fidder et al., 1999. The chemical purity, determined with GC, was 99%. The radiochemical purity was greater than 99% and the specific activity was 53 mCi/mmol.

3.2.2 *Exposure of skin pieces to HD vapor and human skin organ culture*

Human mammary skin was obtained from cosmetic surgery with informed consent of the patient and was exposed to saturated HD vapor at 25 °C using a vapor cup device as described earlier (Mol et al., 1991). The duration of exposure to HD vapor was 5 min. Organ cultures of human skin were maintained as described by Varani et al. (1995). Skin pieces of 0.25 cm^2 were floated with the dermal side down in keratinocyte basal medium (KBM; Cambrex) supplemented with $CaCl_2$ to a final concentration of 1.4 mM (KBMCa; 1 ml medium/well of a 12 well cluster plate) and incubated at 37 °C in an atmosphere of 6% CO_2 in air. Post-exposure incubation was for 48 h, unless otherwise indicated.

BB94 (a gift of British Biotech; according to manufacturer inhibits MMP-1, -2, -3, -7, -9), Ilomastat (a gift of Quick Med Technologies; according to manufacturer inhibits MMP-1, -2, -3, -8, -9) Z-VAD-fmk (Sigma Aldrich Chemie), Z-IETD-fmk (Sigma Aldrich Chemie), Z-LEHD-fmk (Sigma Aldrich Chemie), MMP-2/MMP-9 inhibitor I(Calbiochem; inhibits MMP-2 and MMP-9), inhibitor of MMP-1, -3, -7 and -9 (Inhibitor II; Calbiochem), inhibitor of MMP-1, -2, -3, -7, -13 (Inhibitor III; Calbiochem) and herbimycin A (Sigma Aldrich Chemie) were dissolved in DMSO and diluted in organ culture medium to the desired final concentrations. The final concentration of DMSO was always 1%. Dec-RVKR-cmk (Bachem AG), H-D- Phe-Pro-Arg-cmk (Bachem AG), and TAPI-2 (Calbiochem; according to manufacturer inhibits TACE and MMPs) were directly dissolved into the organ culture medium to the desired final concentrations.

For each condition at least two replicate experiments were performed with n=2 per experiment.

3.2.3 *Human epidermal keratinocyte culture and exposure to HD*

Cultures of HEK were raised from basal keratinocytes, isolated from human mammary skin obtained during cosmetic surgery with informed consent of the patient. In brief, primary epidermal cells were inoculated on a feeder layer of mitomycin C-treated 3T3 mouse fibroblasts in serum-containing medium as described earlier (Mol et al., 1989). When subconfluent, cells were trypsinized and cryopreserved as a stock. For experiments, first passage HEK from cryovials were grown in serum-free keratinocyte growth medium (KGM; Cambrex) at 37 °C in an atmosphere of 6% CO_2 in air. Usually, confluent monolayer cultures were achieved at 7 days after plating.

One day after reaching confluence, HEK were exposed to 0, 100 or 150 μM HD for 30 min at 25 °C. Stock solutions of HD were freshly prepared in dry acetone and diluted immediately before use in KBM to obtain the desired working concentrations. The final concentration of acetone in the incubation medium was 1%.

After exposure, the cells were washed and processed for 2D-PAGE either immediately or after an incubation period of 2, 4, 8, 18, 24, 32 and 42 h at 37 °C in KGM.

To verify whether protein fragments were due to cleavage by caspases, HEK cultures were incubated with KGM containing 20 μM of the caspase-3 and -7 inhibitor Z-DEVD-fmk, or 20 μM of the caspase-6 inhibitor Z-VEID-fmk, or 20 μM of the pancaspase inhibitor Z-VAD-fmk. The caspase inhibitors were added 3 h prior the HD exposure and were present throughout the 24 h post-exposure period.

For exposure of cultured HEK to ^{14}C HD, conditions were comparable with those used for cold HD. The desired HD concentration in KBM was prepared by appropriate dilution of a freshly made ^{14}C HD stock solution in acetone based on the counts it contained. One day after reaching confluence, HEK were exposed to solutions with various concentrations of ^{14}C HD for 30 min at 25 °C. Next, the cells were washed and after a 1 h-incubation period at 37 °C in KGM processed for 2D-PAGE. For radioactivity determinations, samples containing ^{14}C activity were mixed with scintillation fluid (Hionic-Fluor; Packard) and counted in a Tricarb 2500 TR scintillation counter (Packard). In experiments using ^{14}C HD, recovery data for radioactive counts were calculated and found to be 90 % or more.

3.2.4 *Histology*

For histological examination by light microscopy, human skin pieces were fixed overnight at 4 °C in 2% paraformaldehyde in phosphate buffered saline (PBS). They were stored in 70% ethanol until embedding in paraffin. Sections were stained with hematoxylin/eosin and examined by light microscope.

3.2.5 *Immunohistochemistry*

For immunohistochemical examination, human skin pieces were frozen in Tissue-Tek OCT embedding compound (Sakura Finetek) and 5 μm cryostat sections were cut. After our observation that Ln-5 is rapidly broken down in the sections even when they were kept frozen at -20° C, slides with cryosections were processed immediately after cutting. This adaptation of the procedure also eliminated artificial microvesicles that were previously seen in frozen sections that were immunostained for Ln-5. After drying on air at room temperature, sections were fixed in acetone at 4° C for 10 min, and then rinsed in PBS. Endogenous peroxidase activity was quenched in 1.0 % hydrogen peroxide. The immunoperoxidase staining was performed using Neutralite-avidin HRP (Southern Biotech) and 3-amino-9-ethyl-carbazole (AEC) is applied as a chromogenic substrate. Sections were incubated overnight at 4° C with the primary antibodies, followed by 90 min incubation at room temperature with biotinylated goat anti-mouse IgG (Sigma Aldrich Chemie). Monoclonal mouse anti-human laminin-5 (clone GB3; Harlan Sera-Lab) was diluted 1: 300. Monoclonal anti-MMP-2 (clone 75-7F7; Oncogene) was diluted to a final concentration of 0.7 ng/μl. Finally, slides were counterstained with haematoxylin and mounted under a coverslip. Negative controls were performed by omitting the primary antibody during the first incubation.

3.2.6 *In situ zymography*

To demonstrate biological activity of MMP-2, *in situ* gelatin zymography was applied according to Zhang and Salamonsen (2002). In brief, 5 μm cryostat sections of human skin were cut and after drying on air at room temperature, fixed in 10% buffered formalin 4° C for 5 min and then rinsed with cold PBS.

For nuclear counterstaining, 5 μg/ml propidium iodide (Molecular Probes) in PBS was applied on the sections for 8 min. Then, the sections were incubated overnight in the dark with fluorescein- conjugated DQ-gelatin (Molecular Probes) at a concentration of 10 μg/ml in

a mixture of 2% gelatin and 2% sucrose in PBS at 37 °C. The sections were viewed using a fluorescent microscope with a FITC filter.

3.2.7 *2D-PAGE*

Cells were solubilized in 40 mM Tris buffer (pH 8.0) containing 0.5% SDS, and the lysate was boiled immediately for 10 min at 95 °C. After cooling on ice, the lysates were incubated for 2 h at 4 °C with 100 mM DTT, protease inhibitor cocktail (Complete ® Mini, Roche Diagnostics) and DNase I/ RNaseA (Roche Diagnostics). After sonification by four bursts of 5 s, proteins were precipitated in 11% trichloroacetic acid in ice-cold acetone supplemented with 25 mM DTT. Pellets were washed twice with ice-cold acetone and subsequently dissolved in 9.5 M urea, 4% CHAPS, 0.5% IPG buffer and 25 mM DTT. Protein content was determined with the RC/DC protein assay (BioRad). Samples of 150 µg protein were loaded on 13 cm Immobiline IPG strips (4-7) (Amersham Biosciences). Isoelectric focusing was carried out at 20° C to 33.8 kVh on an IPGphor apparatus. Before SDS-PAGE, the focused IPG strips were equilibrated, reduced and alkylated in a buffer containing 6 M urea, 50 mM Tris-HCl, pH 8.8, 2% SDS, 30% glycerol with 10 mg DTT/ml or 25 mg iodoacetamide/ml, respectively. Then, the proteins were separated in the second dimension on homogeneous polyacrylamide gels (10% T). The SDS-PAGE was run at a constant current of 30 mA/gel at 20 °C. Gels were stained by a mass spectrometry compatible silver staining (Yan et al., 2000). Comparative analysis of protein patterns has been performed partially by eye and partially by using ImageMaster 2D Elite software (Amersham Biosciences). For each comparison at least four replicate gels of each sample were used. Changes in protein spots were considered as genuine differences in protein expression when they showed similar qualitative changes in expression in all examined gels originating from one sample.

3.2.8 *Protein identification*

Spots of interest were excised and cut into 1-2 mm^3 pieces. The particles were washed and dehydrated before they were digested with 100 ng trypsine sequencing grade (Promega) in 25 mM NH_4HCO_3 overnight at 37 °C on a shaker (Jensen et al., 1999). The peptides were extracted twice with 5% formic acid and acetonitrile (1:1) and the extracts were pooled and dried in a vacuum centrifuge. Before desalting the peptides were dissolved in 5% formic acid. Desalting was performed with ZipTip microcolumns of C18 (Millipore). Peptide mass fingerprints were determined by matrix assisted laser desorption ionization (MALDI)- time-of-flight (TOF)-MS on a Bruker instrument using α-cyano-4-hydroxycinnamic acid as the matrix. The resulting spectra gave lists of masses that were entered into the Profound (NCBI) program. Search criteria were 0.1 Da mass accuracy and a match of at least four tryptic fragments. The SWISS-PROT database (http://www/expasy.ch/sprot/) was used for additional information on pI, MW and amino acid composition of the identified proteins.

3.2.9 *Determination of HD- protein adduct formation*

Following separation of proteins by 2D-PAGE, ^{14}C labeled proteins on gels were visualized by direct autoradiography. Briefly, gels were dried by air (GelAir Dryer; Biorad) and exposed to the phosphor side of an Imaging Screen-K according to the instructions of the manufacturer (Biorad) for four weeks. Screens were analyzed on the Molecular Imager® FX Pro Plus according to the instructions of the manufacturer (Biorad). Proteins that are bearing ^{14}C HD-adducts are localized at sites that showed darkening on the autoradiogram. To identify

these proteins, corresponding spots were excised from parallel gels that were stained by silver for protein visualization and submitted to mass spectrometric identification.

3.3 Results

3.3.1 *Effectiveness of potential therapeutic agents to prevent microvesication*

Control human skin cultured for 48 h has a normal appearance (*Figure 1A*). Exposure of human skin pieces to saturated vapor of HD at 25 °C for 5 min results in clear epidermal damage with dyskeratotic cells with pyknotic nuclei at 16 hr of post-exposure incubation in KBMCa and in microvesication after a culture period of 48 h (*Figures 1B-1D*). Epidermal-dermal separation is prevented when during post-exposure incubation broad spectrum hydroxamate-based inhibitors of MMPs are present in the culture medium. In the presence of BB94, Ilomastat, TAPI-2 or MMP inhibitor III full protection against HD-induced microvesication can be achieved. The respective lowest effective concentrations for each of these compounds are given in *Table 1*. The suppressive effect of these MMP inhibitors on microvesication is illustrated by the histological sections shown in *Figures 2A-2D.* In addition, the epidermis and dermis of the skin are not torn apart in the presence of 500 μM furin inhibitor (*Figure 2E*). MMP inhibitor II showed improvement at the used concentrations, however full protection was not observed with 40 μM (*Figure 2F*). Increasing concentrations of inhibitor of uPA reduce microvesication but full protection is not achieved at highest concentration of 1 mM (*Figure 2G).* The tyrosine kinase inhibitor failed to suppress microvesication at the highest concentration of 10 μM (*Figure 2H*). Only MMP2/MMP9 inhibitor appeared to be toxic to control human skin and is therefore not suitable for therapy (results not shown).

To investigate the role of apoptosis in the development of microvesication, the organ culture medium was supplemented with an inhibitor of all caspase enzymes, Z-FAD-fmk. The inhibitor was added immediately at the start of the post-exposure incubation, and full blockade of microvesication is observed with concentrations of 10 μM Z-FAD-fmk and higher. In addition, an obvious reduction of epidermal cell death is observed (*Figures 3A-B*). Elimination of microvesication was also achieved when 10 μM caspase-8 inhibitor or 10 μM caspase-9 inhibitor were added to the culture medium immediately after HD exposure (*Figures 3C-D*). When higher concentrations of the caspase-8 inhibitor are used, not only protection against microvesication is observed, but also against nuclear condensation of epidermal cells. The protective effect of 100 μM caspase-8 inhibitor is shown in *Figure 3E.* This effect is not seen when using 100 μM caspase-9 inhibitor (*Figure 3F*).

To investigate how long time is allowed between HD exposure and the application of BB94 to the organ culture medium, without loosing the protective effect of the inhibitor, BB94 is added to the organ culture medium in a final concentration of 4 μM at 0, 8, 14, 18 and 24 h after the start of the post-exposure incubation. In *Figure 4A* it is shown that BB94 can be added as late as 18 h after exposure to HD, without loosing its inhibiting effect on epidermal-dermal separation. Similarly, 10 μM pancaspase inhibitor is added to the organ culture medium at 0, 2, 4, 6, 10, 14, 18 and 24 h after the start of the post-exposure incubation. In *Figure 4B* is shown that pancaspase inhibitor can be added as late as 6 h after exposure to HD, with a similar inhibiting effect on epidermal-dermal separation and on apoptosis as when added immediately after exposure. When the pancaspase inhibitor is added after 10 h (*Figure 4C*), the microvesication can still be prevented, but the effect on nuclear condensation of epidermal cells is diminished. Application of pancaspase inhibitor at 14 h post-exposure has no therapeutic effect (*Figure 4D*).

To see whether the effects of an MMP inhibitor and an apoptosis inhibitor are additive, BB94 and pancaspase inhibitor were added simultaneously to the post-exposure incubation medium. The histological observation shows that the effect is equal to that when pancaspase inhibitor alone is used (result not shown).

3.3.2 *The involvement of MMP-2 and Ln-5 in HD-induced microvesication*

To investigate whether changes in MMP-2 and Ln-5 expression take place during the development of microvesicles, control human skin and skin pieces that were exposed for 5 min to HD vapor were examined immunohistochemically at 4, 8, 16, 24 and 48 h of post-exposure incubation. The immunochemical staining pattern for Ln-5 shows that Ln-5 is markedly present as a thick line along the epidermal-dermal junction in fresh control skin (*Figure 5A*). No changes occur in the expression of Ln-5 in control skin during the culture period of 48 h (*Figure 5B*). In skin that was exposed to HD no reduction of the expression of Ln-5 was seen at the various time points that were examined during post-exposure incubation (not shown). Overt microvesication is observed at 48 h, but Ln-5 is still abundantly present and the thickness of the line is equal to that in control skin at 48 h (*Figure 5C*). Obviously, the anti-Ln-5 staining is confined to the floor of the blisters. TAPI-2 or pancaspase inhibitor had no effect on the expression of Ln-5 (*Figures 5D and 5E*).

MMP-2 is expressed in the epidermis and dermis of unprocessed human skin (*Figure 6A*). The enzyme is present at the cellular membranes of all epidermal cells, however no staining is observed under the basal cells at the SBM (*Figure 6B*). During the culture period of 48 h the expression profile of MMP-2 in control skin does not change (*Figures 6C, 6E and 6G*). In skin that was exposed to HD, the expression of MMP-2 at the surfaces of the cells in the lower part of the epidermis has become irregular at 16 h post-exposure (*Figure 6D*). At 24 h and 48 h, only incidental staining of MMP-2 at the cell membranes of the lower epidermal cell layers is visible (*Figures 6F and 6H*).

The MMP-2 antibody binds to the inactive as well as to the active form of the enzyme. To determine whether the expressed MMP-2 is active, *in situ* zymography was applied using gelatin as a substrate. Gelatinase activity is present in the dermis and in all epidermal layers except for the stratum corneum in fresh uncultured skin (*Figure 7A*). During organ culture gelatinase activity is enhanced in the epidermis of control skin at 4 h and 24 h (*Figures 7B and 7H*), whereas medium gelatinase activity is seen at 8 and 16 h (*Figures 7D and 7F*). At 48 h gelatinolytic activity is low (*Figure 7J*). In HD-exposed skin gelatinolytic activity seems to remain equal to that in uncultured skin during post-exposure incubation for 24 h (*Figures 7C, 7E, 7G and 7I*). No enhanced gelatinase activity is seen along the SBM. At 48 h the fluorescent signal has faded out, like in unexposed skin (*Figure 7K*).

3.3.3 *Analysis of the mechanism of action of HD by a proteomic approach*

Comparative qualitative analysis of gels obtained at 18, 24, 32 and 42 h of incubation following exposure to 0, 100 or 150 µM HD demonstrated several alterations in protein expression that were associated with exposure to HD. The silver stained protein expression pattern of HEK exposed to 100 µM HD obtained at 24 h post-exposure is taken as typical. Results have been described in detail in the annual reports (Mol, 2003, 2004). Briefly, in this protein profile, several spots are observed that are related to HD exposure. Most of them are seen in the area with pI 4.5 – 5.5 and MW 20 -30 kDa. Protein patterns of control and HD-treated HEK at 24 h post-exposure that are found to be typical for this area are shown in *Figure 8.* The protein spots that are indicated with arrows are qualitative treatment-dependent alterations. These difference spots have been subjected to peptide mass fingerprinting

resulting in the identification of twelve of them, listed in *Table 2*. Except for two, the spots were degradation products of the cytoskeletal keratins 14, 16 and 17 that are present in undifferentiated cultured HEK. Spots **b, c, g, h** and **i** are C- and N- terminal fragments of type I keratin 14 (K14). Peptide mass fingerprinting of spots **a, e** and **f** revealed that they are C– and N-terminal fragments of type I K17. Spots **d** and **k** were identified as C- and N-terminal fragments of type I K16. Finally, spots **l** and **m** have been identified as isoforms of heat shock protein (HSP)27.

The presence in the culture medium of the pancaspase inhibitor Z-FAD-fmk, the caspase -3 and -7 inhibitor Z-DEVD-fmk, or the caspase-6 inhibitor Z-VEID-fmk during post-exposure incubation prevented the appearance of the keratin fragments. The HSP27 spots **l** and **m** remain expressed in the presence of caspase inhibitors, indicating that their appearance is independent of caspase activity (*Figure 9*).

To analyse the formation of ^{14}C HD-protein adducts in cultured HEK, 2D-PAGE was applied on cell lysates of HEK that were exposed to 125 μM ^{14}C HD. *Figure 10A* shows an autoradiogram of proteins that contain ^{14}C HD-adducts. Comparison with a silver-stained gel that was run under the same conditions (*Figure 10B*) shows that only a limited number of proteins from the total protein pool of HEK bear ^{14}C HD-adducts. This result indicates that adduct formation occurs selectively. Keratins appear to be predominantly alkylated by HD (*Table 3*). Interestingly, high molecular weight aggregates of keratins were found on top of the gel (spots 1 and 2). Endoplasmin (grp94/gp96), actin and galectin-7 were identified as three non-keratin proteins that are alkylated by HD. Spot 17 was not identified in this experiment but from earlier studies we know that this is stratifin (Mol, 2003).

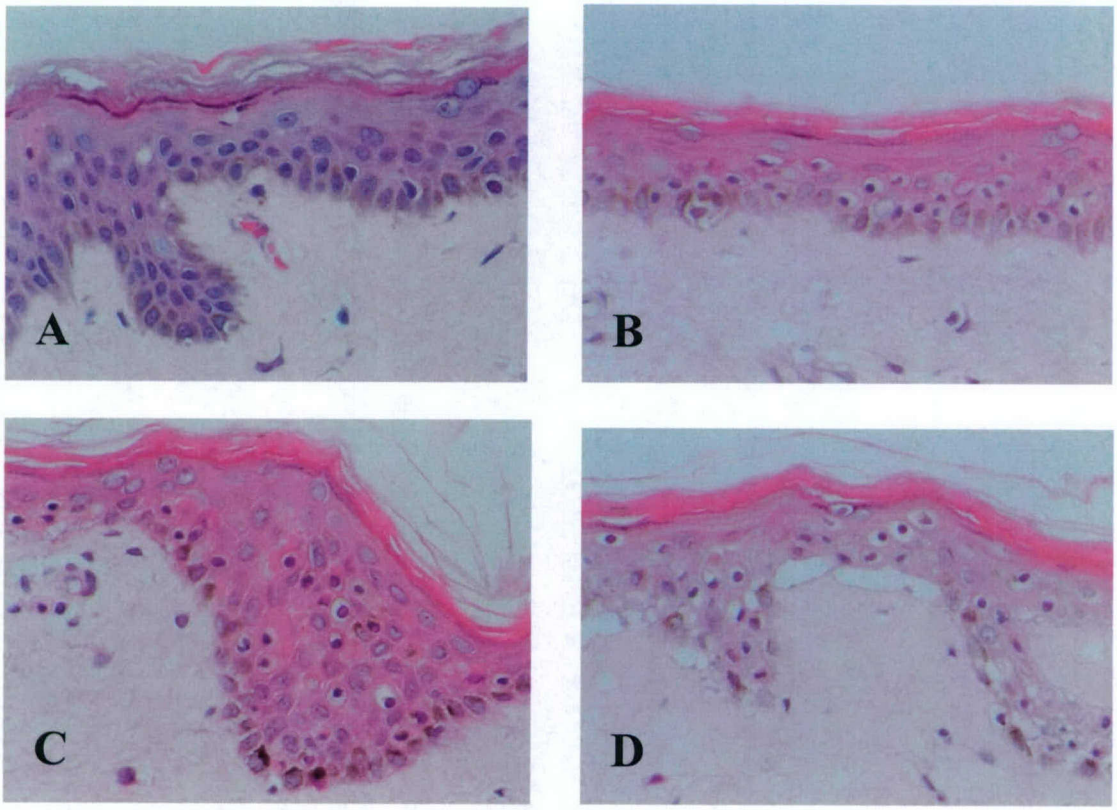


Figure 1. *Development in time of damage in human skin that was exposed for 5 min to saturated vapor of HD at 25 °C and subsequently organ cultured for 48 h in KBMCa.*
To examine skin morphology during the pathogenesis of microvesication, paraffin sections were stained with hematoxylin/eosin. Skin samples were collected of control skin (**A**) and of HD-exposed human skin at 16 h (**B**), 24 h (**C**) and 48 h (**D**) of post-exposure organ culture. Cell death can be observed in a few cells in the epidermis at 16 h after exposure. Massive epidermal cell damage and microvesication develop between 24 and 48 h of post-exposure incubation.

Table 1. *Potential therapeutic agents to reduce microvesication.*
Compounds were tested for their therapeutic value in reduction of microvesication induced by exposure for 5 min to saturated HD vapor of human skin in an *ex vivo* model. Compounds were added at the start of the 48 h period of post-exposure incubation in KBMCa.

Compound	Concentration range (μM)	Lowest effective concentration (μM)	Toxicity on human skin
BB94	0.2 -10	4	no
Ilomastat	0.02 -200	100	no
MMP2/MMP9 inhibitor	30-650	n.d.	yes, ≥65 μM
MMP inhibitor II	0.2 - 40	≥ 40	no
MMP inhibitor III	0.2 – 20.0	15	no
TAPI-2	1-50	20	no
Dec-RVKR-cmk	25- 500	500	no
H-D-FPR-cmk	1-1000	≥ 1000	no
Z-VAD-fmk	0.1 - 200	10	no
Z-IETD-fmk	5 - 100	25	no
Z-LEHD-fmk	5 - 100	50	no
Herbimycin A	0.1 – 10	≥ 10	no

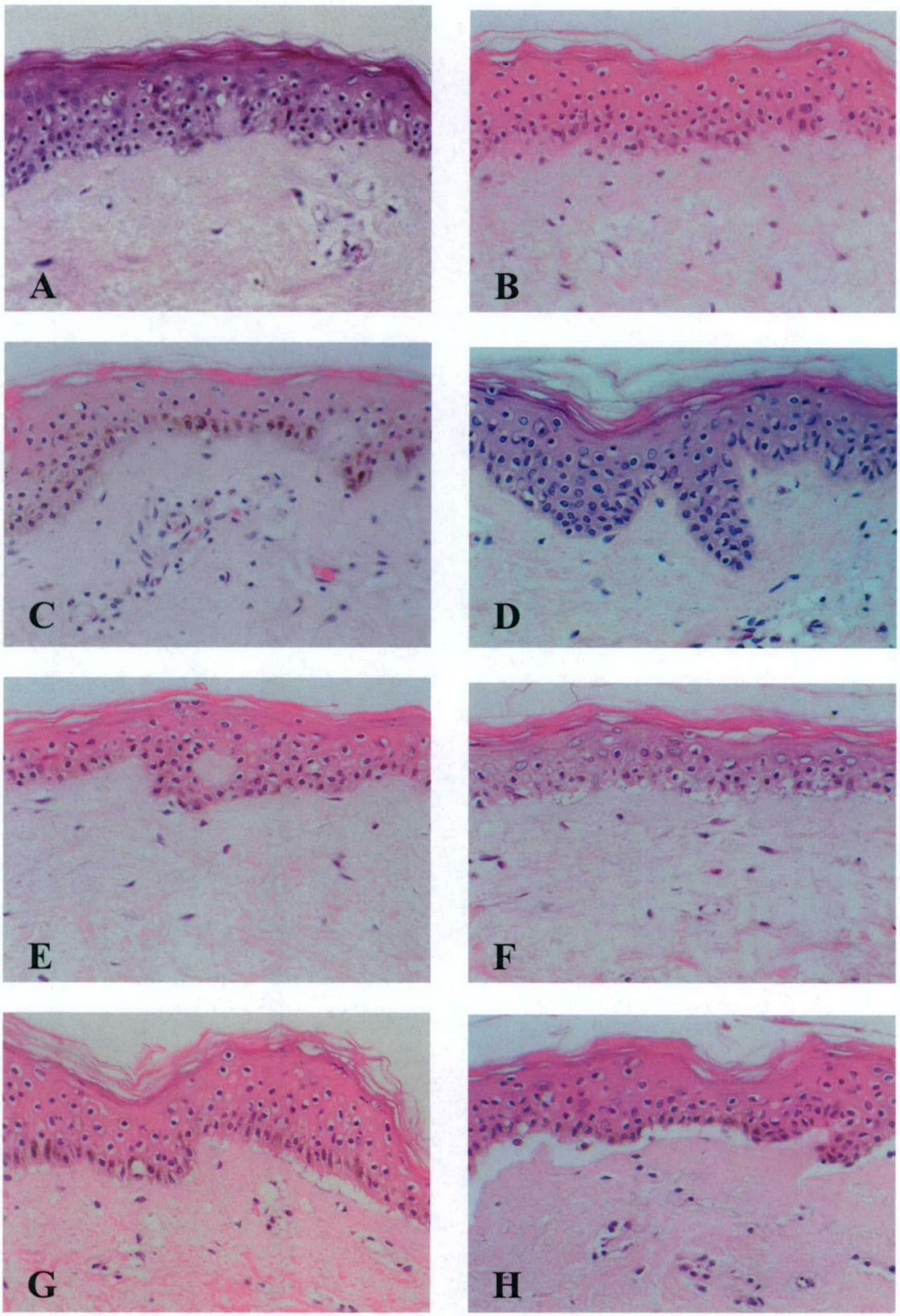


Figure 2. *Effects of various inhibitors on microvesication in human skin that has been exposed to saturated vapor of HD for 5 min.* After exposure, skin is organ cultured in KBMCa for 48 h in the presence of (**A**) 4 μM BB94, (**B**) 20 μM TAPI-2, (**C**) 15 μM MMP inhibitor III, (**D**) 100 μM Ilomastat, (**E**) 500 μM furin inhibitor, (**F**) 40 μM MMP inhibitor II, (**G**) 1 mM uPA inhibitor or (**H**) 10 μM herbimycin A. Microvesication is fully blocked in the presence of BB94, TAPI-2, MMPinhibitor III, Ilomastat or furin inhibitor.

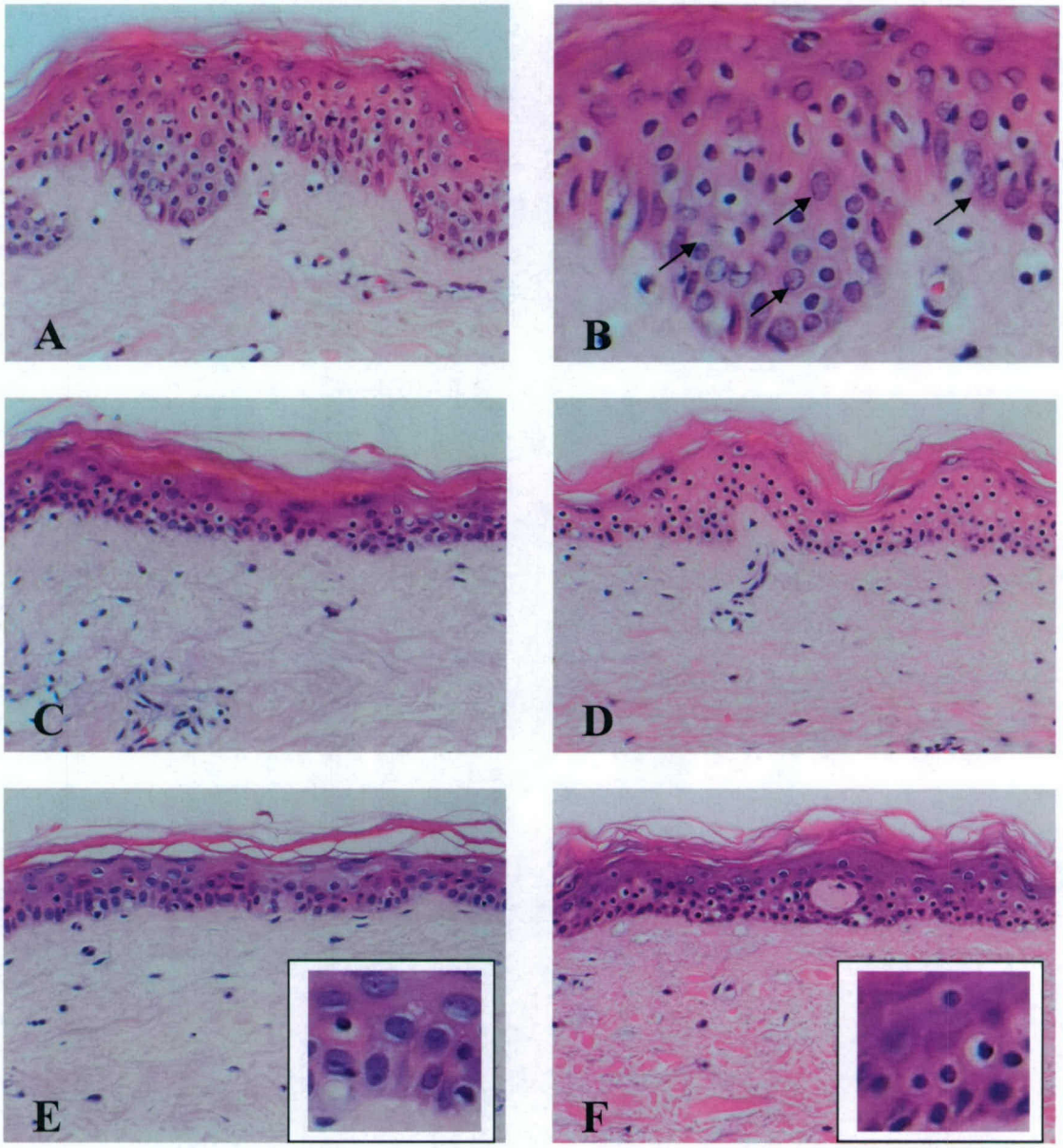


Figure 3. *Effects of caspase inhibitors on microvesication in human skin that has been exposed to saturated vapor of HD for 5 min..*
During post-exposure incubation the following inhibitors were added to the medium (**A**) 10 μM pancaspase inhibitor, (**B**) detail of (A) showing cells without nuclear condensation, arrows, (**C**) 10 μM caspase -8 inhibitor, (**D**) 10 μM caspase-9 inhibitor, (**E**) 100 μM caspase-8 inhibitor, or (**F**) 100 μM caspase-9 inhibitor. Application of 10 μM of each inhibitor eliminates microvesication (**A, C, D**). Moreover, with the use of 10 μM pancaspase inhibitor or 100 μM caspase-8 inhibitor nuclear condensation of epidermal cells is remarkably reduced (**B, E-insert**). This protective effect is not seen when using 100 μM caspase-9 inhibitor (**F-insert**).

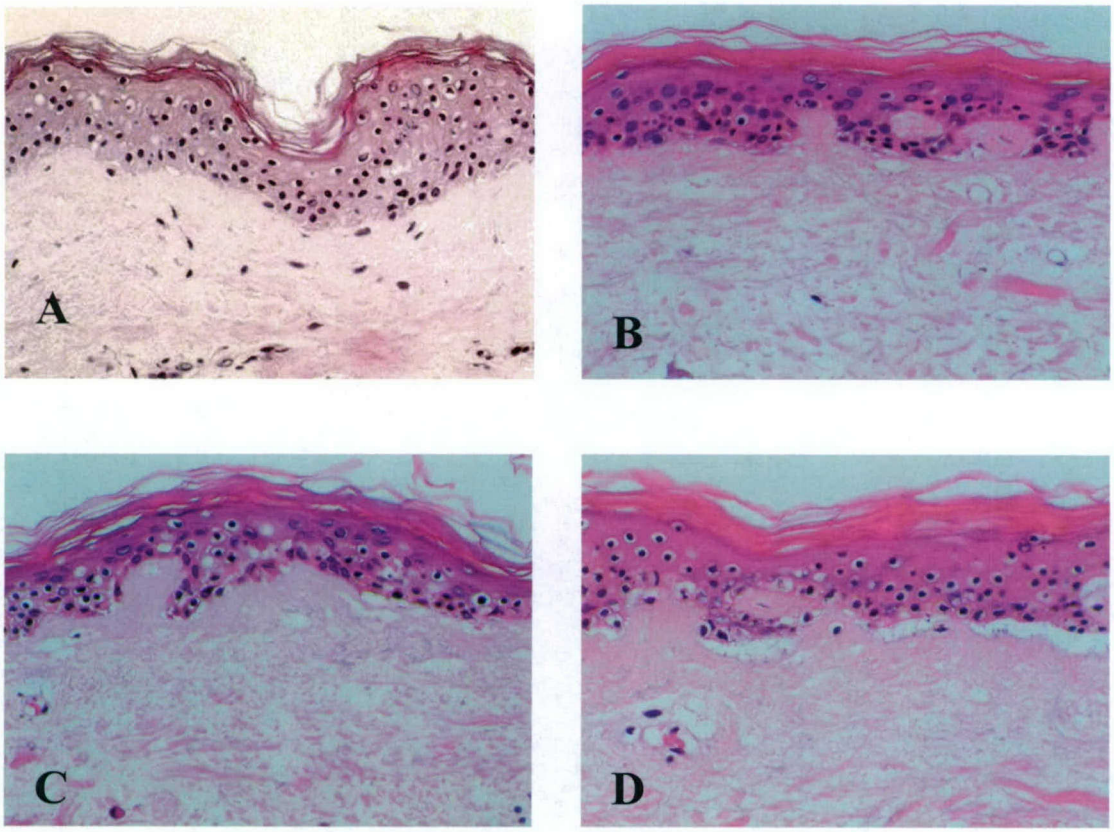


Figure 4. *Effects of delayed application of BB94 or pancaspase inhibitor on microvesication in human skin that has been exposed to saturated vapor of HD for 5 min.*
A full protective effect is observed when 4 μM BB94 is added to the culture medium as late as 18 h after exposure (**A**). 10 μM pancaspase inhibitor was added to the culture medium (**B**) at 6 h, (**C**) at 10 h or (**D**) at 14 h after exposure. A full protective effect is still observed when pancaspase inhibitor is added 6 h after HD exposure (**B**). When the pancaspase inhibitor is added after 10 h, microvesication can still be prevented, but the protective effect on nuclear condensation of epidermal cells is attenuated (**C**). Microvesication can not be blocked when addition of pancaspase inhibitor is postponed till 14 h post-exposure (**D**).

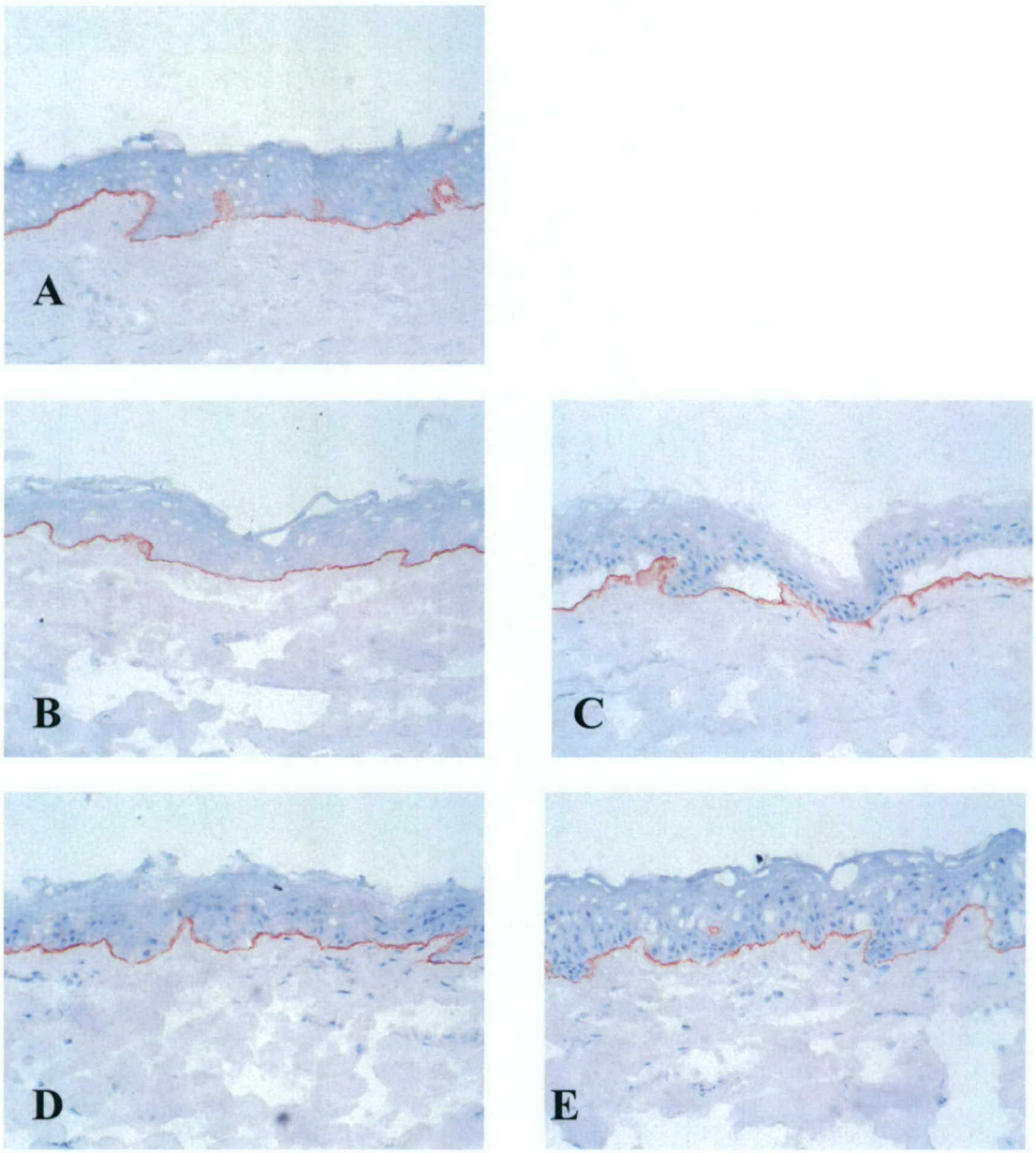


Figure 5. *Immunohistochemical staining of laminin-5 in human skin that has been exposed to saturated vapor of HD for 5 min.*
The expression of laminin-5 is examined in frozen sections of fresh control skin (**A**), control human skin at 48 h of post-exposure organ culture (**B**) and HD-exposed human skin at 48 h of post-exposure organ culture (**C**). The staining intensity of laminin-5 is not changed in HD-exposed skin. When epidermis is detached of dermis, laminin-5 remains with the microblister base. To prevent microvesication, the medium of HD-exposed skin was supplemented with TAPI-2 (**D**) or pancaspase inhibitor Z-FAD-FMK (**E**). The staining intensity of laminin-5 did not change in the presence of each of these inhibitors.

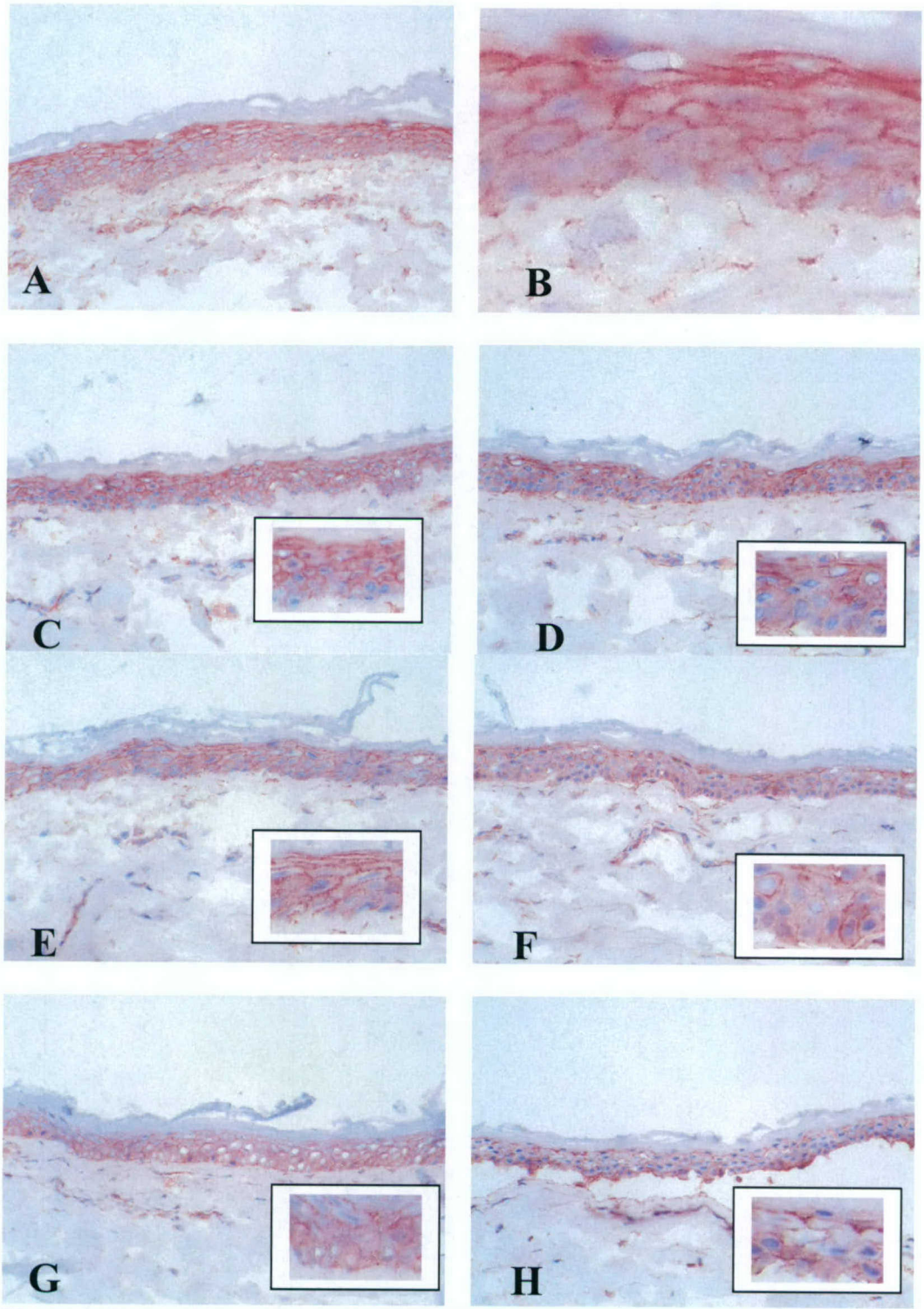
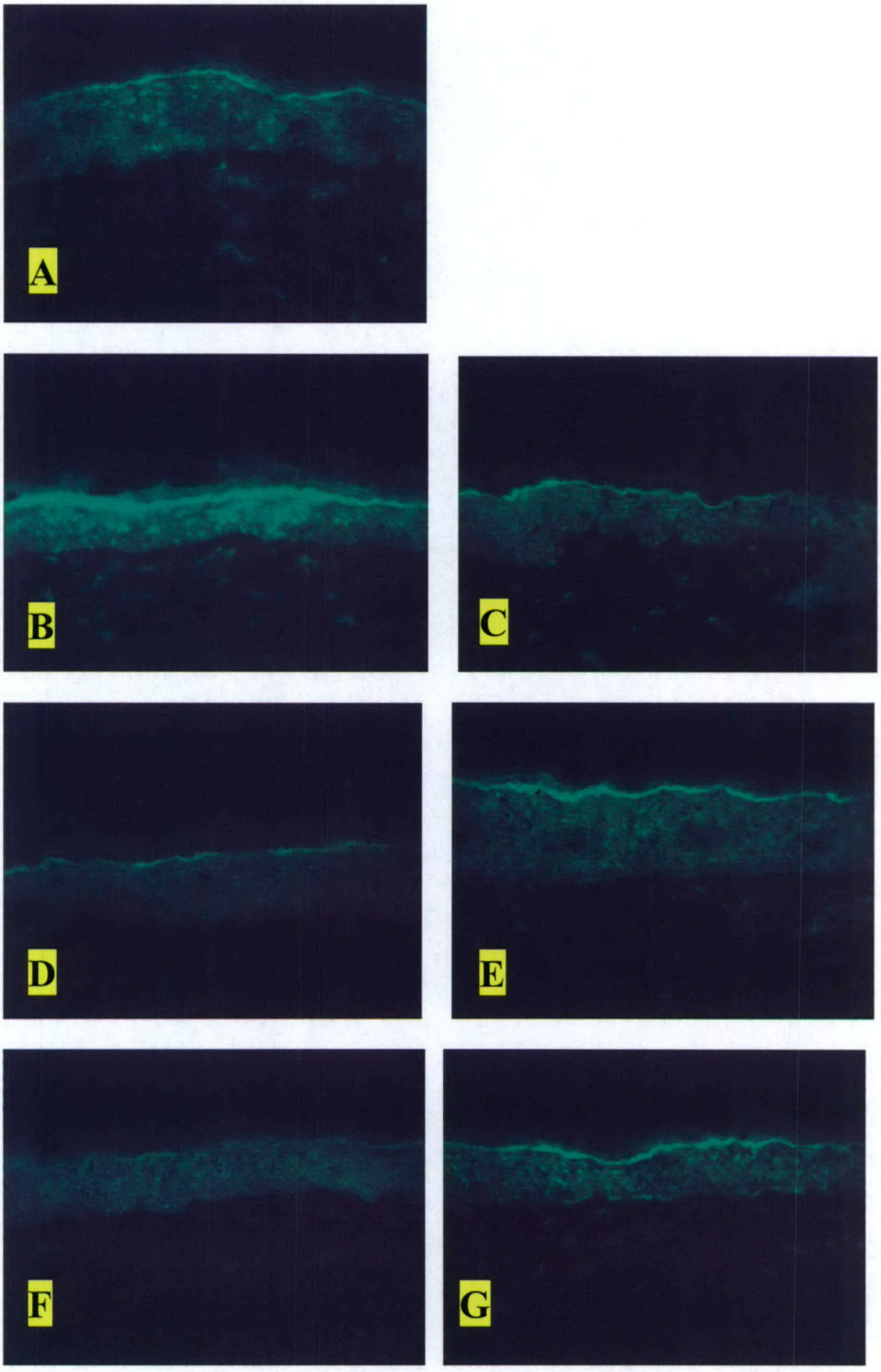


Figure 6. *Immunohistochemical staining of MMP-2 in human skin that has been exposed to saturated vapor of HD for 5 min.*

The expression of MMP-2 was examined in frozen sections of fresh control skin (**A; B** is enlargement of A), control human skin at 16 h (**C**), 24 h (**E**) and 48 h (**G**) of organ culture and HD-exposed human skin at 16 h (**D**), 24 h (**F**) and 48 h (**H**) of post-exposure organ culture. The expression profile of MMP-2 in control skin does not change. In HD-exposed skin, the expression of MMP-2 at the surfaces of the cells in the lower part of the epidermis becomes irregular during post-exposure incubation (see inserts for details). No staining is observed under the basal cells.


A
B
C
D
E
F
G

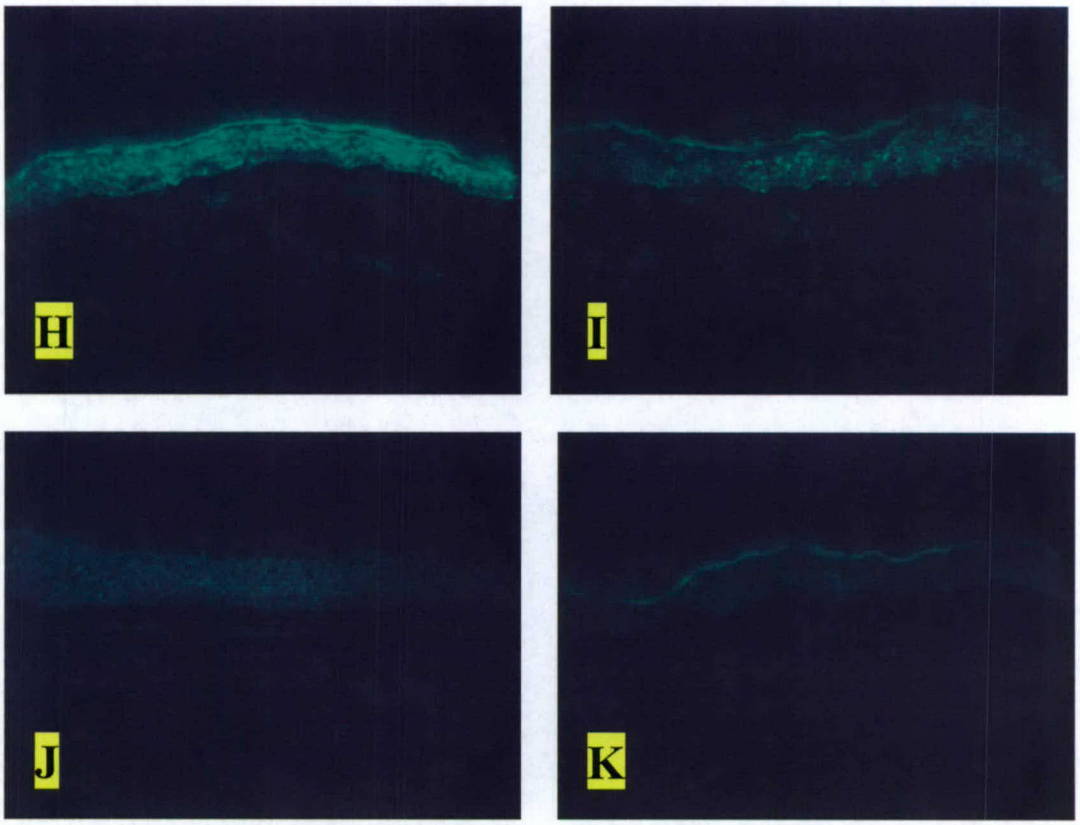


Figure 7. *Gelatinase activity measured by in situ zymography in human skin that has been exposed to saturated vapor of HD for 5 min.*
Gelatinase activity was examined in frozen sections of control skin (**A, B, D, E, F, H, J**) and HD-exposed skin (**C, E, G, I, K**) at various time points during organ culture. Gelatinolytic activity was determined in fresh uncultured skin (**A**), and in skin that was cultured for 4 h (**B, C**), 8 h (**D, E**), 16 h (**F, G**), 24 h (**H, I**) and 48 h (**J, K**). Gelatinase activity is present in uncultured control skin. Enhancement of gelatinase activity in control skin is seen at 4 h and 24 h of organ culture. In HD-exposed skin gelatinolytic activity remains equal to that in uncultured skin for 24 h. After 48 h of organ culture the fluorescent signal has faded out in control skin as well as in HD-exposed skin.

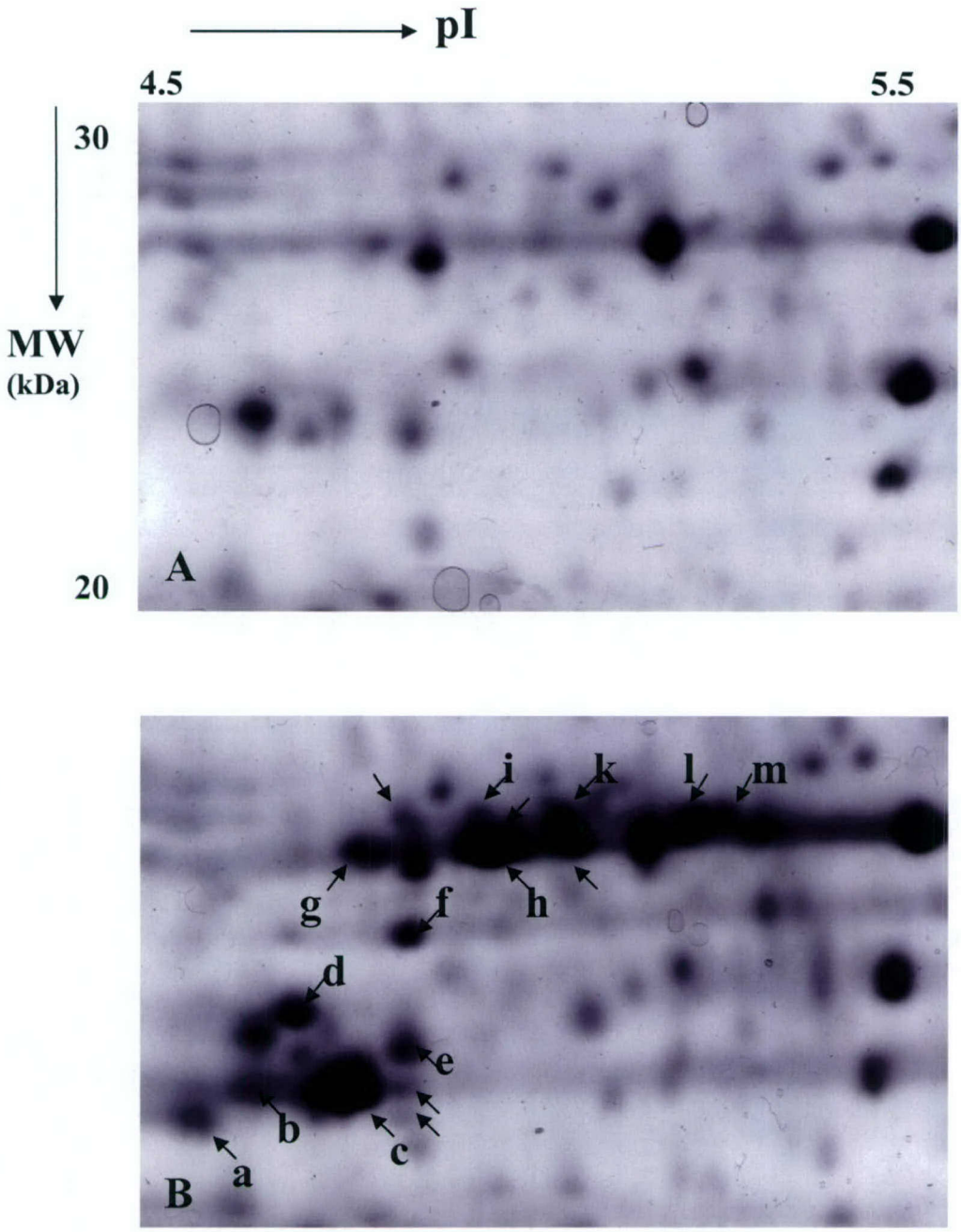
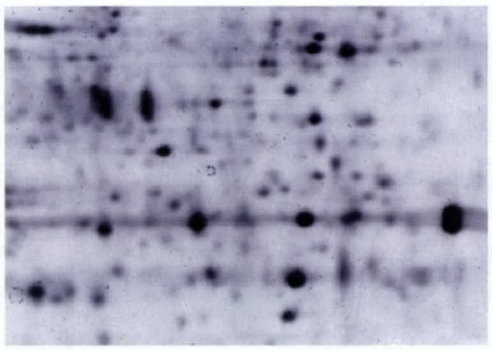


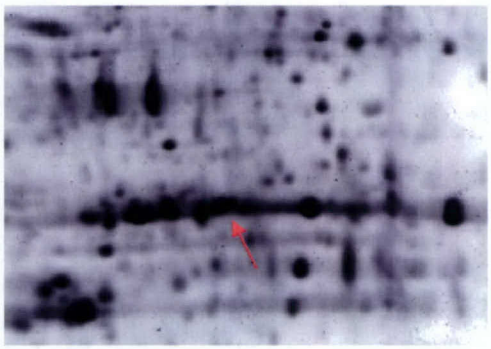
Figure 8. *Differential expression of proteins from control HEK and HD-exposed HEK.* Protein expression in lysates of cultured HEK, collected at 24 h after exposure to 0 (**A**) or to 100 μM HD (**B**). The area shown is confined to pI 4.5-5.5 and MW 20 -30 kDa. The protein spots that are indicated with arrows are qualitative HD-treatment related alterations. Spots annotated with **a** through **m** have been identified and their names are listed in *Table 2*.

Table 2. *List of proteins that were found to be HD-exposure related spots.*
Proteins were identified by MALDI/TOF/MS analysis. Letters correspond to spots indicated in *Figure 8B*. Values for MW and pI are according to the SWISSPROT database.

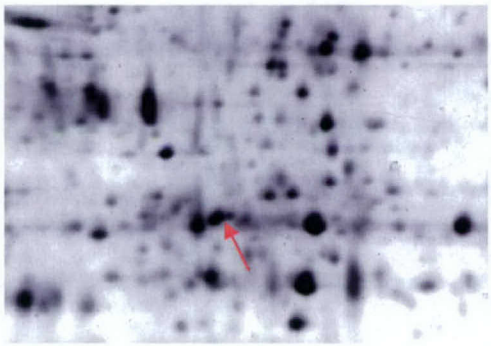
Spot	Protein name	Swiss Prot entry (intact protein)	Theor. MW (kDa)/pI
a	Keratin 17, C-terminal fragment	Q04695	22.0/5.0
b	Keratin 14, C-terminal fragment	P02533	22.9/5.1
c	Keratin 14, C-terminal fragment	P02533	22.9/5.1
d	Keratin 16, C-terminal fragment	P08779	22.5/4.8
e	Keratin 17, C-terminal fragment	Q04695	22.0/5.0
f	Keratin 17, N-terminal fragment	Q04695	25.9/5.0
g	Keratin 14, N-terminal fragment	P02533	28.5/5.1
h	Keratin 14, N-terminal fragment	P02533	28.5/5.1
i	Keratin 14, N-terminal fragment	P02533	28.5/5.1
k	Keratin 16, N-terminal fragment	P08779	28.6/5.2
l, m	Heat shock protein 27	P04792	22.8/6.0



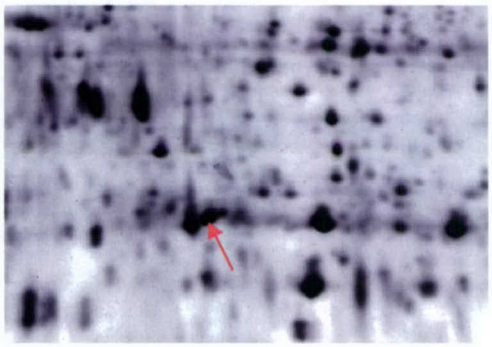
0 μM HD



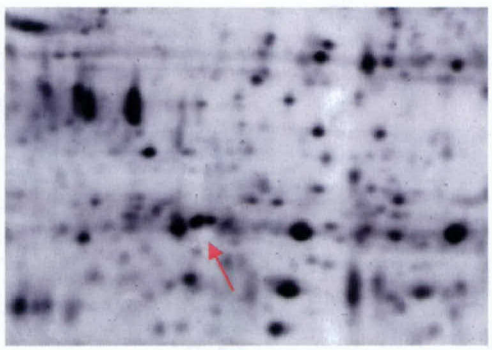
100 μM HD



100 μM HD + 20 μM pancaspase inhibitor

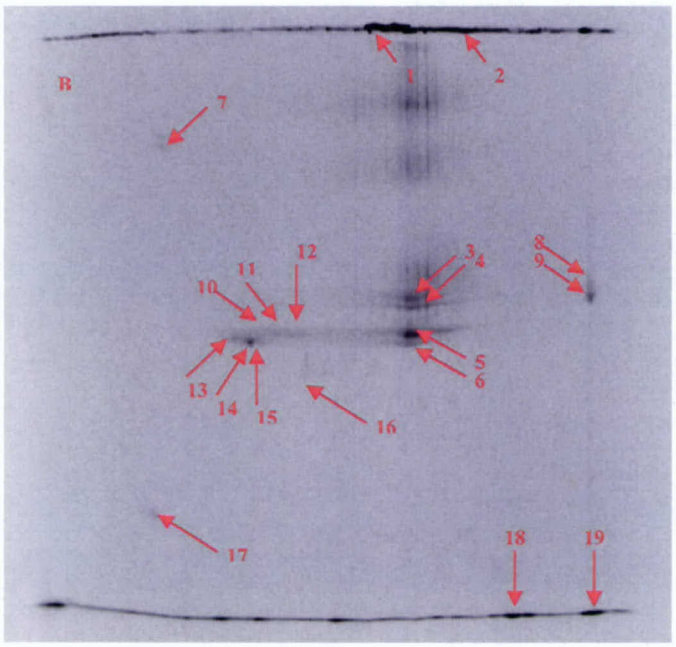


100 μM HD + 20 μM caspase-3 inhibitor

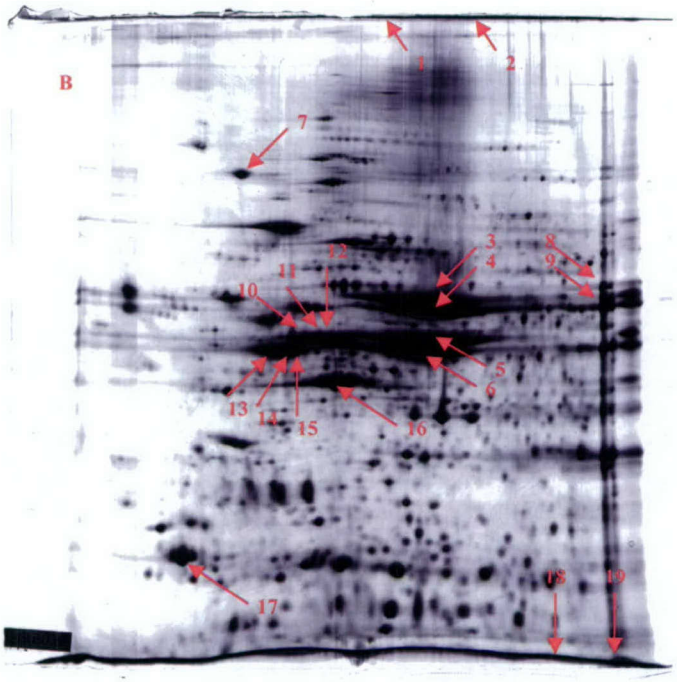


100 μM HD + 20 μM caspase-6 inhibitor

Figure 9. *Effects of various caspase inhibitors on the cleavage of cytoskeletal keratins in HEK that were exposed to 100 μM HD.*
HEK were incubated with caspase inhibitors during 3 h pre-exposure and during the next 24 h post-exposure. In the presence of the caspase inhibitors no keratin fragments were formed. The HD-exposure related expression of HSP27 (red arrows) was not affected by caspase inhibition.



A



B

Figure 10. *Analysis of the formation of HD-protein adducts in HEK.*
(**A**) Autoradiogram of 2D-PAGE gel showing ^{14}C-HD-adducted proteins in the total cell lysate of cultured HEK that were exposed to 125 µM ^{14}C-labelled HD. (**B**) Parallel gel showing the silver stained 2D-PAGE pattern from the same cell lysate. The gels cover proteins with pI 4-7 (horizontal axis) and MW 20-200 kDa (vertical axis). A limited number of proteins have bound adducts of HD. The numbers indicate the proteins that were cut from the silver stained gel for identification. Results are listed in *Table 3*.

Table 3. *Identification of proteins that have formed HD-adducts.*
Proteins that contain ^{14}C HD –adducts were identified by MALDI-TOF/MS analysis. Numbers correspond to spots indicated in *Figure 10*. Values for MW and pI are according to the SWISSPROT database.

Spot	Protein name	Theoretical MW (kDa)/pI
1	Keratin 5 Keratin 6	54.6/5.6 60.2/8.0
2	Keratin 6 Keratin 14	60.3/8.1 51.9/5.1
3	Keratin 16	51.5/5.0
4	Keratin 6	60.2/8.1
5	Keratin 14	51.9/5.1
6	Keratin 17 Keratin 16 Keratin 14	48.8/5.1 51.5/5.0 51.9/5.1
7	Endoplasmin (grp94/gp 96)	90.3/4.7
8	Keratin 6 Keratin 5	60.2/8.1 54.6/5.6
9	Keratin 6	60.2/8.1
10	Keratin 14	51.9/5.1
11	Keratin 14	51.9/5.1
12	Keratin 14	51.9/5.1
13	Keratin 16	51.5/5.0
14	Keratin 16	51.5/5.0
15	Keratin 16	51.5/5.0
16	Actin (beta?)	41.3/5.6
17	Not identified.	--
18	Not identified	--
19	Galectin-7	15.1/7.0
B	Blank	

3.4 Discussion

3.4.1 *Effectiveness of potential therapeutic agents to prevent microvesication*

The presence of the hydroxamate-based metalloproteinase inhibitors BB94, Ilomastat, TAPI-2 and MMP inhibitor III in the organ culture medium of human skin pieces that were exposed to HD vapor abolishes the typical HD-induced microvesication. On the other hand, MMP inhibitor II could not fully protect against microvesication. Since MMP inhibitor II, in contrast to the other inhibitors, does not inhibit MMP-2 according to the manufacturer's information, this observation supports the idea that this enzyme is involved in epidermal-dermal detachment following HD exposure of human skin *ex vivo*. However, based on our results on MMP-2 expression and activity in HD-exposed human skin (see sections 3.3.2 and 3.4.2.), it is considered to be unlikely that MMP-2 causes microvesication by proteolysis of SBM proteins in the *ex vivo* model. For BB94, Ilomastat and TAPI-2 broad inhibitory activity has been described against several other MMPs than MMP-2, f.e., MT1-MMP, as well as against TACE (Labrousse et al., 2002; Iida et al., 2004; Deryugina et al., 2002; Contin et al., 2003). Based on the protective effects on microvesication that were obtained with these inhibitors, it is supposed that MT1-MMP and TACE will be involved in HD-induced microvesication. The observation that inhibition of furin is also effective in preventing epidermal-dermal separation supports this assumption, since furin is involved in the maturation of MT1-MMP and TACE (Yana and Weiss, 2000; Peiretti et al., 2003). The incomplete protective effect that was obtained with uPA inhibitor indicates that the uPA/plasmin system might be only partially involved in microvesication in the human skin *ex vivo* model. To obtain stronger evidence for the involvement of MT1-MMP and TACE more specific inhibitors should be tested than the ones with limited selectivity that were used during this study. At this time, there is major interest in developing specific inhibitors for MMPs which are known to be involved in pathological processes such as arthritis, cancer and heart and lung diseases (Puerta and Cohen, 2004; Rao, 2005). In addition, dual inhibitors of MMPs and TACE are highly sought as potential therapeutics, because inhibition of TACE might not only reduce degradation of membrane proteins, but also suppress inflammatory pathways initiated by TNFα (Kottirsch et al., 2002; Trifilieff et al., 2002). This type of inhibitors might be beneficial in treatment of HD-damage in skin, eyes and lungs, because of the inflammatory reaction that accompanies HD-induced pathogenesis in these tissues.

The role of MMPs/TACE in HD-induced microvesication was at first assumed to cause weakening of the SBM by loosening of structural proteins such as Ln-5 or collagen XVII from the anchoring hemidesmosomes. However, evidence has become available that these members of the metalloprotease family are involved in shedding of many important cell surface proteins resulting in the release of biologically active soluble factors (for review: Comaglio et al., 2003). For example, TACE-mediated shedding of EGF receptor ligands represents a critical event in keratinocyte migration. Upon ligand activation, the EGF receptor increases the phosphorylation levels of the integrinβ4 cytoplasmic domain both on tyrosine residues, through intermediate stimulation of Fyn activity (Mariotti et al., 2001) and serine residues, through a protein kinase C-dependent pathway (Rabonivitz et al., 2004). Enhanced phosphorylation of integrinβ4 leads to hemidesmosome disassembly. Taken together, MMPs/TACE are probably crucial in the disassembly of hemidesmosomes by cleavage of their ligands, as well as by indirectly causing phosphorylation of integrinβ4. Inhibition of these enzymes preserves epidermal-dermal attachment by protection of the hemidesmosomal structure.

The observation that application of BB94 as late as 18 h after exposure to HD vapor is still effective in blocking microvesication provides evidence that proteolytic activity by MMPs/TACE is negligible until that time point. This idea is supported by the finding in this study that only sparse epidermal cell death is seen at 24 h after HD exposure and that massive cell death and microvesication develop between 24 and 48 h post-exposure.

The present study shows that inhibition of caspase-8 provides considerable protection against nuclear condensation of epidermal cells as well as microvesication in human skin pieces that were exposed to HD vapor. The obtained results are similar to those seen using pancaspase inhibitor. In contrast, the inhibitor of caspase-9 is incapable to defend epidermal cells against nuclear condensation, but, surprisingly, yet leads to the prevention of microvesication. This remarkable observation means that caspases that are specifically downstream of caspase-9 probably cleave molecules that control epidermal-dermal attachment. The fact that inhibition of caspase-8 greatly diminishes nuclear condensation in keratinocytes while inhibition of caspase-9 does not, indicates that inhibition of caspase-8 shuts down all caspase activity. Therefore, caspase-8 is apical to caspase-9 in the apoptotic cascade that is triggered in epidermal cells following exposure to HD. This conclusion is in accordance with results of Rosenthal et al. (2003), who demonstrated that caspase-8 is activated prior to caspase-9 (1 *versus* 4 h) in keratinocytes that were exposed to HD. Furthermore, they showed that Fas and its ligand were induced and that a FADD dominant negative mutant could completely inhibit apoptosis caused by HD. This predominant role for the extrinsic Fas/FasL pathway and caspase-8 in apoptosis of keratinocytes coincides with data from the literature (Takahashi et al., 2001; Kim et al., 2003; Marconi et al., 2004).

The observation that the addition of pancaspase inhibitor to the organ culture medium as late as 6 h after exposure to HD still protects epidermal cells against cell death, means that till that time caspases are almost not activated and the apoptotic process is not yet started. This in agreement with results of Chen et al., (2000), who found that pancaspase inhibitor added 8 h after genotoxic insult prevented reduction of ATP and massive release of cytochrome c from the mitochondria. A delay of 6-9 h in substantial activation of caspases is also seen following ultraviolet irradiation of keratinocytes (Sitailo et al., 2002).

3.4.2 *The involvement of MMP-2 and Ln-5 in HD-induced microvesication*

Ln-5 is a main adhesion protein of epithelial cells that links hemidesmosomes with the basement membrane. It was assumed that a reduced expression of Ln-5 might contribute to HD-induced pathogenesis of microvesication. However, our results show that when microvesicles are present, the expression of Ln-5 along the blister base is equal to that in control skin without microvesication. It is concluded that HD exposure does not affect the expression of Ln-5 in the human skin *ex vivo* model and that reduced expression of Ln-5 is not a causative factor for microvesication. The results presented in this report are in contrast with those described earlier (Mol, 2004). Since our previous staining results appeared to be not consistent, artificial loss of antigen was presumed to occur. It was found that Ln-5 is rapidly broken down in the cryosections even when they were kept frozen at -20° C. Therefore, the procedure has been adapted and slides with cryosections were processed immediately after cutting. Since then, staining results were satisfactory. The staining of Ln-5 at the dermal side of the blister indicates that the connection of Ln-5 with dermal proteins is still intact, but that the bond with the integrins probably has been disrupted.

Immunostaining of MMP-2 in fresh uncultured human skin shows membrane-bound expression of the enzyme in all layers of the epidermis, except for the stratum corneum. This seems not to correspond to articles that report absence of immunostaining for MMP-2 in

normal skin (Fleischmajer et al., 2000). An explanation for this difference might be that in our case several hours have passed between the surgery in the hospital and the moment of processing in the laboratory, while in other reports biopsies are taken and processed immediately there after. The expression of MMP-2 that is observed in our skin specimens might be a response towards injury.

The observation that the expression of MMP-2 in HD-exposed skin decreases with time in the lower epidermal cell layers does not fit with our hypothesis that MMP-2 is actively involved in microvesication. It was supposed that for participation of MMP-2 in microvesicaton, expression of the enzyme should at least be found in the area of the SBM. Neither enhanced enzymatic activity was seen in this region using gelatin *in situ* zymography. Based on these results and on earlier studies of the role of MMP-2 in HD-induced pathogenesis (Mol, 2000), it is concluded that MMP-2 does not play a role in HD-induced microvesication in the human skin *ex vivo* model.

3.4.3 *Analysis of the mechanism of action of HD by a proteomic approach*

The application of a proteomic approach in the search for mechanisms of action of HD has provided valuable new insights. First, the elucidation of a time-dependent appearance of fragments of K14, K16 and K17 in HEK pointed towards proteolytic breakdown of intermediate filaments by caspases. Since these enzymes are strictly associated with apoptosis, these observations leaded us to investigate the role of apoptosis in the formation of microvesicles in the human skin *ex vivo* model (see section 3.4.1). Indeed, inhibition of caspase activity had beneficial effects on microvesication and epidermal cell morphology.

Apart from the discovery of caspase-6 dependent keratin fragments, HSP27 has been identified as HD-exposure related protein. A phosphorylated isoform of this protein is detected in the protein profile of HD-exposed keratinocytes. HSP27 is one of several proteins that become phosphorylated by p38 mitogen-activated protein (MAP) kinase in response to stress (Larsen et al., 1997). This finding might indicate that p38 MAP kinase plays a role in the cellular response upon HD-exposure. MAP kinase pathways are important mechanisms by which extracellular signals can lead to alterations in gene expression (Han et al., 1997). Recently, Dillman et al., (2004) have demonstrated that inhibition of the p38 MAP kinase signaling causes down regulation of cytokine release in response to HD. In general, HSP27 plays a major role in cellular response to stress and might even block apoptosis (Arrigo, 1998; Charette et al., 2000; Pandey et al., 2000; Concannon et al., 2003). Focus of research on the role of this protein in cellular response to HD might be important.

To complement our prior studies on the formation of HD-protein adducts (Mol, 2000) we repeated the exposure of HEK to ^{14}C-labelled HD. The obtained autoradiogram was similar to that seen in the study at that time. Protein identification has now confirmed our previous assumption that mostly keratins are targets for alkylation by HD. HD-adducts appear to be formed with the acidic type II as well as with the basic type I keratins. High molecular weight aggregates containing K5/K6 and K6/K14 were found on top of the gel. It can not be stated whether these aggregates result from imperfect lysis of keratin pairs or from intermolecular protein cross-linking by HD. Arguing for the latter option is the demonstration by Dillman et al., (2003) that HD induces aggregation of K5 and K14 in keratinocytes. The effect of alkylation of keratins by HD on functioning of the cytoskeleton is unclear. It is tempting to speculate that collapse of the keratin filament network of HEK following exposure to HD (Werrlein and Madren-Whalley, 2003) is a result of keratin alkylation. Only a very small number of proteins other than keratins are bearing a ^{14}C HD-adduct. Actin, endoplasmin, stratifin and galectin-7 have been identified in this study as other target proteins for alkylation by HD. The impact of alkylation of these proteins on the toxic mechanism of

action of HD is largely unknown. However, with this information available, new perspectives on the toxic pathway of HD are generated.

4 Key research accomplishments

- This study shows clear evidence that HD-induced microvesication in *ex vivo* human skin is dependent on apoptosis and metalloprotease activity. Therefore, inhibition of the apoptotic process and of metalloprotease activity will be a therapeutic way to eliminate the formation of blisters in HD casualties.

- HD-induced microvesication in *ex vivo* human skin is produced by members of the metzincin subfamily of metalloproteases, such as MT1-MMP and TACE, because pharmacologic inhibition of MMPs/TACE activity by broad spectrum hydroxamate-based compounds, such as BB94, Ilomastat, TAPI-2 and MMP Inhibitor III, fully prevents microvesication in human skin pieces that were exposed for 5 min to saturated vapor of HD. In addition, the furin inhibitor dec-RVKR-cmk blocks HD-induced microvesication in *ex vivo* human skin.

- Activation of MMPs/TACE in HD-exposed human skin is not an early event in the pathogenic process because BB94, a representative of the broad spectrum hydroxamate-based inhibitors, can fully prevent microvesication if added to the culture medium as late as 18 h after exposure.

- Microvesication in HD-exposed human skin *ex vivo* is not caused by MMP-2 activity or by reduced expression of the adhesion molecule Ln-5.

- HD-induced microvesication in *ex vivo* human skin is mediated by members of the apoptosis-related family of caspases because inhibition of all caspases, as well as inhibition of the two initiator caspases, caspase-8 and caspase-9, stops HD-induced microvesication in human skin pieces that were exposed for 5 min to saturated vapor of HD.

- Pancaspase inhibitor and caspase-8 inhibitor, but not caspase-9 inhibitor, greatly diminished the number of dyskeratotic cells with pyknotic nuclei in HD-exposed human skin.

- Apoptosis is a key event in the mechanism of action of HD and caspase-8 is the most apical caspase in the apoptotic process of keratinocytes in the human skin *ex vivo* model.

- Full activation of caspases in HD-exposed human skin does not occur immediately after insult by HD, because pancaspase inhibitor can fully prevent microvesication and maintain epidermal cell morphology, if added to the culture medium as late as 6 h after exposure. Microvesication, but not changes in epidermal cell morphology, is stopped even when pancaspase inhibitor is given 10 h post-exposure.

- The application of a proteomic approach in the search for mechanisms of action of HD has generated valuable new clues. By means of this non-hypothesis-driven approach the involvement of apoptosis and of phosphorylated HSP27 was discovered. The latter finding points also towards p38 MAP kinase as an important enzyme in the mechanism of action of HD. Furthermore, keratins were identified as a bulk target for

the formation of HD-protein adducts. Only few proteins other than keratins are alkylated by HD.

- Exposure of HEK to HD (100 or 150 μM) causes cleavage of K14, K16 and K17 by caspase-6.

5 Reportable outcome

Posters and symposium proceedings:

Mol MAE, van den Berg RM. ((2003) Inhibitors of matrix metalloproteases block sulfur mustard-induced epidermal-dermal separation. *Poster presentation at 7th Medical Chemical Defense Conference, Munich, Germany.*

Mol MAE, van den Berg RM, van Dijk C, de Jong AL. (2003) Proteomics as a strategy to study the mechanistic toxicology of sulfur mustard. *Proceedings of the 2003 Meeting of NATO TG004, Medicin Hat, Canada.*

Mol MAE, van den Berg RM, van Dijk C, de Jong AL. (2004) Sulfur mustard causes caspase-mediated cleavage of cytoskeletal keratins. *Proceedings of the Bioscience 2004 Medical Defense Review, Hunt Valley MD, USA.*

Mol MAE, van den Berg RM, Chau LF. (2004) Therapeutic relevance of inhibitors of MMPs or of caspases in HD-induced injury in the ex vivo human skin model. *Proceedings of the Bioscience 2004 Medical Defense Review, Hunt Valley MD, USA.*

Mol MAE, van den Berg RM, Chau LF. (2005) Effective medical countermeasures against sulfur mustard exposure of skin and eye are closer than ever before. *Proceedings of the 2004 Meeting of NATO TG004, Hradec Kralove, Czech Republic.*

6 Conclusions

The results of the present study using the human skin *ex vivo* model, point towards two key aspects in the mechanism of action of HD that are being involved in the development of microvesication: the activity of enzymes of the metalloprotease family and the onset of apoptosis. Therefore, elimination of these causal conditions for blister formation must be used as a regimen to cure skin lesions in HD casualties. The observation that pancaspase inhibitor and MMP inhibitor can fully prevent microvesication in the human skin *ex vivo* model if applied 6 h and 18 h, respectively, after exposure to HD opens perspectives for non-urgent cure of HD casualties. The application of both classes of inhibitors in *in vivo* studies with, f.e., the hairless guinea pig is the next step to be taken in our itinerary to provide therapeutic agents against HD injury of the skin.

Based on the current results for Ln-5, the working hypothesis that limited availability of SBM proteins due to a disturbed balance between production and degradation of these proteins is causative for the destabilization of the SBM is no longer tenable. In addition, no proof has been found in favor of the supposed role of MMP-2 in the degradation of SBM proteins. The role of metalloproteases is probably more subtle than merely the degradation of SBM proteins. Their involvement will be rather pertained to shedding of biologically active proteins from the cell surface. Through receptor activation, kinase signaling pathways will be triggered which influence cell attachment to the SBM by means of phosphorylation.

The proteomic approach used in this study has proven to be a valuable tool. The obtained results have broadened our perception of the mechanism of action of HD at the molecular level. The appearance of keratin fragments following exposure of HEK to HD and subsequently, their absence in response to the use of caspase inhibitors have established the mechanistic involvement of apoptosis in the HD-induced cascade of cellular events. Additionally, the expression of a more acidic isoform of HSP27, indicating phosphorylation of this protein, has raised the supposition that p38 MAP kinase plays a role in the cellular response upon HD-exposure. Here, another hint is found for the role of kinase signaling pathways in the cellular response to HD. Finally, keratins have been identified as a bulk target for the formation of HD-protein adducts. Their alkylation may have serious consequences for the integrity of the cytoskeleton and cellular function.

The current explorations of causing mechanisms and potential lead compounds for therapy in the human skin *ex vivo* model have yielded general mechanistic themes that will also apply to injury induced by HD in eyes and lungs. Therefore, this study has contributed to progress in the search of medical countermeasures against HD-damage in multiple organs.

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8 Appendices

8.1 List of abbreviations

ADAM	A disintegrin and metalloprotease
AEC	3-amino-9-ethylcarbazol
CHAPS	3-[3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DNase	Deoxyribonuclease
DTT	1,4-Dithiotreitol
EGF	Epidermal growth factor
FADD	Fas-associated death domain
FITC	Fluorescein isothiocyanate
HD	Sulfur mustard
HRP	Horseradishperoxidase
HSP	Heat shock protein
HEK	Human epidermal keratinocytes
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
K14	Keratin 14
KBM	Keratinocyte basal medium
kDa	Kilodalton
KGM	Keratinocyte growth medium
Ln-5	Laminin-5
MALDI-TOF	Matrix- assisted laser desorption/ionization – time of flight
µM	Micromolar
mM	Millimolar
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
PBS	Phosphate buffered saline
p38MAPkinase	p38 mitogen-activated protein kinase
pI	Isoelectric point
RC/DC	Reductant compatible/detergent compatible
RNase	Ribonuclease
SBM	Skin basement membrane
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TACE	Tumor necrosis factor-alpha converting enzyme
TNFα	Tumor necrosis factor-alpha
Tris	Tris(hydroxymethyl)-amino-methane
uPA	Urokinase-type plasminogen activator

8.2 List of suppliers

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Oncogene, Boston, MA, USA

Packard, Groningen, The Netherlands

Promega, Leiden, The Netherlands

Quick Med Technologies, Gainesville, FL, USA

Roche Diagnostics, Almere, The Netherlands

Sakura Finetek Europe, Zoeterwoude, The Netherlands

Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands

Southern Biotechnologies Associates, Birmingham, AL, USA

8.3 Personnel

People that contributed to the realization of this three-year research are:

At TNO Defence, Safety & Security, location Rijswijk (formerly TNO Prins Maurits Laboratory:

- S.W. Alblas, technical assistent biochemistry
- Hammer, technical assistant histology
- Fidder, technical assistant organic chemistry
- E.S. Milder-Enacache, technical assistent biochemistry
- R.M. van den Berg, technical assistent biochemistry
- van Dijk, technical assistent biochemistry
- L.F. Chau, technical assistant histology
- A.L. de Jong, technical assistant mass spectrometry
- M.A.E. Mol, principal investigator
-

At Biomedical Primate Research Centre, Rijswijk

- W. Collignon, technical assistant histology

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